Crystal structure of adenosine kinase from *Toxoplasma gondii* at 1.8 Å resolution

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Abstract

Human infection with *Toxoplasma gondii* is an important cause of morbidity and mortality. Protozoan parasites such as *T. gondii* are incapable of de novo purine biosynthesis and must acquire purines from their host, so the purine salvage pathway offers a number of potential targets for antiparasitic chemotherapy. In *T. gondii* tachyzoites, adenosine is the predominantly salvaged purine nucleoside, and thus adenosine kinase is a key enzyme in the purine salvage pathway of this parasite. The structure of *T. gondii* adenosine kinase was solved using molecular replacement and refined by simulated annealing at 1.8 Å resolution to an *R*-factor of 0.214. The overall structure and the active site geometry are similar to human adenosine kinase, although there are significant differences. The *T. gondii* adenosine kinase has several unique features compared to the human sequence, including a five-residue deletion in one of the four linking segments between the two domains, which is probably responsible for a major change in the orientation of the two domains with respect to each other. These structural differences suggest the possibility of developing specific inhibitors of the parasitic enzyme.

Keywords: adenosine kinase; crystal structure; purine metabolism; *Toxoplasma gondii*

The obligate intracellular protozoan parasite *Toxoplasma gondii* is distributed worldwide with up to 50% infection rates in some parts of the United States (Nash et al., 1998). In hosts with intact immunity, infection is usually benign (McCabe & Remington, 1988). However, persons with defective cell-mediated immunity and neonates with perinatally acquired disease are susceptible to severe and potentially fatal infections (Seigel et al., 1971; Britt et al., 1981; Luft & Remington, 1988; Grant et al., 1990; Dannemann et al., 1992; Luft & Castro, 1992). In particular, *T. gondii* has emerged as a major opportunistic pathogen in patients with AIDS, especially in the central nervous system. Treatment of toxoplasmosis is complicated by severe toxicity associated with the currently available chemotherapeutic agents. There is a continuing need to define chemotherapeutic targets and develop new drugs for this pathogen.

One feature of *T. gondii* that distinguishes it from its human host is its inability to synthesize purines de novo (Schwartzman & Pfefferkorn, 1982; Krug et al., 1989). Unlike its mammalian host, *T. gondii* predominantly salvages purine precursors via adenosine kinase, the enzyme that converts adenosine to AMP, from which all other purine nucleotides can be synthesized. Adenosine is incorporated into the nucleotide pools of *T. gondii* at a 10-fold higher

rate than any other purine base or nucleoside (Schwartzman & Pfefferkorn, 1982). Two pathways for the conversion of adenosine to nucleotides appear to exist, one involving adenosine kinase and the other involves hypoxanthine-guanine phosphoribosyltransferase. Studies with adenosine kinase-deficient mutants of *T. gondii* suggest that in the wild-type parasite, the adenosine kinase pathway is predominant (Schwartzman & Pfefferkorn, 1982). Adenosine kinase, which is the most active enzyme in the purine salvage pathway, is therefore a key enzyme.

Deficiency of adenosine kinase activity is not lethal to *T. gondii*, but inhibition of adenosine kinase activity would be expected to inhibit purine salvage. In this regard, it is noteworthy that purine analogues are potent inhibitors of *T. gondii* in vivo. For example, adenine arabinoside (Ara-A), a nucleoside analogue in which adenine is linked to arabinose rather than ribose (or deoxyribose), is a potent and selective inhibitor of *T. gondii* in human fibroblast cultures. Adenosine kinase is considered to be the target enzyme for Ara-A, as some Ara-A resistant mutants possess <0.1% of the adenosine kinase activity of the wild-type parasite (Pfefferkorn & Pfefferkorn, 1976). Unfortunately, few attempts have been made to exploit this striking difference between toxoplasma and its host (Pfefferkorn & Pfefferkorn, 1976; Luft, 1986).

Because of the critical role adenosine kinase plays in the regulation of adenosine and adenylate levels in tissues and the clinical applications of adenosine kinase inhibitors, the mammalian enzyme has been extensively studied (Miller et al., 1979a, 1979b;

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Palella et al., 1980; Yamada et al., 1980; Chang et al., 1983; Fisher & Newsholme, 1984; Mimouni et al., 1994; Singh et al., 1996; Spychara et al., 1996; McNally et al., 1997). Recently, the structure of human adenosine kinase (HAK) complexed with two molecules of adenosine was determined (Mathews et al., 1998). Interestingly, the overall structure of human adenosine kinase is similar to the structure of ribokinase (Sigrell et al., 1998), but it shows little similarity to other nucleoside or nucleotide kinases. In contrast, very little is known about invertebrate adenosine kinase. The enzyme from *Leishmania donovani* is the only other parasitic enzyme that has been isolated and characterized (Datta et al., 1987). *T. gondii* adenosine kinase (TAK) has been partially characterized in terms of its ligand binding properties (Iltzsch et al., 1995). However, characterization has been complicated by the difficulty of isolating and purifying the active enzyme. We have over-expressed recombinant TAK, crystallized it in the presence of adenosine, and determined its structure at 1.8 Å resolution. Comparison of the structure of TAK with HAK and ribokinase (RK) shows several important differences.

Results

Quality of the structure

Table 1 is a summary of the refinement parameters. Superposition of the *R*-factor and free *R*-factor curves with theoretical curves for different mean positional errors gives estimated errors of 0.22 and 0.25 Å, respectively (Luzzati, 1952). Deviations from ideal stereochemical values are 0.005 Å for bond lengths, 1.2° for bond angles, 22.6° for dihedral angles, and 0.78° for improper angles. Comparison of the stereochemistry of the structure with other structures solved at the similar resolution using PROCHECK (Laskowski et al., 1993) showed that all of the main-chain and side-chain

parameters were better than the mean. A Ramachandran plot showed that 92.5% of the residues were in most favored regions, and all but three others were in additional allowed regions. Two residues (Arg83 and Ser159) are in disallowed regions, and Ser198 is in a generously allowed region with conformational angles close to the first residue in a type II' turn. Residues Arg83 and Ser198 correspond to Gln78 and Ser198 in HAK, which are also in disallowed regions. All three residues have good electron density. A plot of χ_1 vs. χ_2 showed no unfavorable conformations. The average temperature factor is 26.8 Å². The highest temperature factors in the molecule occur at the N-terminus, the hairpin loops 133–136 and 290–295, the putative ATP-binding loop 304–314, and the residues adjacent to the gap between residues 237 and 246.

Description of the structure

TAK contains two domains comprised of 12 α -helices (31–36, 54–58, 70–82, 98–109, 154–157, 175–186, 200–205, 207–214, 224–233, 249–264, 316–329, and 334–348), 14 β -strands (15–18, 22–28, 44–46, 64–68, 88–95, 113–119, 126–132, 135–141, 163–167, 192–196, 219–222, 274–278, 282–289, and 295–300), and three short stretches of 3_{10} helix (59–61, 143–147, and 168–172), and a short turn (50–51). Domain 1 includes residues 1–22, 68–125, and 143–363, while domain 2 includes residues 23–67 and 126–142 (Figs. 1, 2). The overall structure of TAK is similar to HAK, and individual domains in TAK superimpose closely with the corresponding domains in HAK (Fig. 3). Comparison of domains 1 and 2 of TAK with the corresponding domains in HAK gives root-mean-square (RMS) differences between C α carbons of 0.91 and 0.80 Å, respectively. The largest differences occur in the following regions: residues 96–111, 118–122, 174, 295–299, 303–314, and 357–358 in domain 1 and residues 47–53, 62, and 133 in domain 2. Most of these residues are in loops or turns, including the putative ATP binding loop (residues 303–314). This loop is rather poorly defined and has some of the highest temperature factors in the structure. Presumably this is due to the absence of substrate in the binding site, which probably stabilizes the loop. Residues 295–299 are in a β -strand, which has moved slightly because of the longer hairpin loop in TAK compared to HAK (Fig. 3).

Even though the individual domains of TAK and HAK are very similar, the orientation of the two domains with respect to each other is quite different (Fig. 3). The two domains are connected by four polypeptide segments (residues 19–21, 62–63, 120–125, and 142–146). Comparison of the TAK and HAK sequences adjacent to the fourth segment shows a five-residue deletion in TAK compared to HAK, which results in a large movement of domain 2 with respect to domain 1. In HAK, the folding of domain 2 over domain 1 creates the active-site cavity, and the interactions between these domains are stabilized by an adenosine molecule. However, this cavity is much larger in TAK, and the buried surface area at the interface between domains is correspondingly smaller (Fig. 4).

As expected, the structure of TAK is also similar to RK, although less so than to HAK. Comparison of the two domains of TAK with the corresponding domains in RK gives RMS deviations (RMSDs) between C α carbons of 1.17 and 0.83 Å, respectively. As with HAK, the individual domains are very similar, but the orientation of the two domains with respect to each other is quite different. In fact, the orientation of the two domains is more similar in HAK and RK than in TAK with either of the other two proteins.

Table 1. Refinement statistics for *T. gondii* adenosine kinase

Resolution range (Å)	46.8–1.80
Number of reflections	33,069
Completeness (%)	97.9
<i>R</i> value	0.214
Free <i>R</i> value	0.233
Highest resolution shell (Å)	1.80–1.91
Number of reflections	4,982
Completeness (%)	88.9
<i>R</i> value	0.273
Free <i>R</i> value	0.293
Estimated coordinate error (Å)	0.22
Deviations from ideality	
Bond lengths (Å)	0.005
Bond angles (°)	1.2
Dihedral angles (°)	22.6
Improper angles (°)	0.78
Temperature factor refinement	
Mean <i>B</i> value (Å ²)	26.8
RMSD main-chain bond (Å ²)	0.96
RMSD main-chain angle (Å ²)	1.53
RMSD side-chain bond (Å ²)	1.52
RMSD side-chain angle (Å ²)	2.29

Fig. 1. Sequence comparisons of TAK and adenosine kinases from different organisms. The alignment of TAK and HAK is based on the three-dimensional structure comparison. Numbering follows the sequence of TAK. Secondary structures are shown as cylinders (helices) and arrows (strands). Residues in TAK involved in interactions (hydrophobic and/or hydrogen bonding) with adenosine are shown by filled triangles. GenBank accession numbers for the various AK sequences are *T. gondii* (AF128275), human (U50196), rat (U57042), *Caenorhabditis elegans* (Z81107), and *Saccharomyces cerevisiae* (P47143). The figure was drawn with ALSCRIPT (Barton, 1993).

Adenosine binding site

One adenosine molecule is present in this structure, and it corresponds to the adenosine site in HAK and the ribose site in RK. Superposition of the 12 residues from HAK that participate in binding adenosine to the corresponding residues in TAK generally shows differences of 0.2 to 0.9 Å. The major differences in TAK occur in residues Asp24, Cys127, Leu138, and Thr140, which are displaced by 3.3 to 6.1 Å relative to HAK. All of these residues are in domain 2, which has moved away from domain 1 in the TAK structure.

Unfortunately, the electron density for adenosine is poorly defined (Fig. 5), and refinement was problematic. Group refinement of the occupancies for adenosine atoms gave a value of 0.56, although the density for the adenine moiety is better than for the ribose moiety. The adenine moiety refined to a position with pro-

tein contacts very similar to the adenine portion of adenosine in the HAK structure (Fig. 6). It is slightly tilted with respect to the adenine moiety in the HAK structure, possibly because movement of Tyr169 requires tilting of the adenine ring to maintain the base stacking relationship.

The ribose moiety has a considerably different conformation and orientation with respect to adenine and to the residues that bind to ribose in the HAK structure. The glycosidic torsion angles (Donohue & Trueblood, 1960) for adenosine in TAK and HAK are -88.0° and -123.3° , respectively. While the ribose rings in HAK and RK adopt the unusual O4'-endo conformation, the ribose ring in TAK has the more common C3'-endo conformation. This suggests that the ribose in TAK is not in the preferred conformation for enzymatic activity. Furthermore, of the five protein-ribose hydrogen bonds that are present in HAK and RK, only two are preserved in TAK. In particular, there are no direct protein contacts

Fig. 2. Ribbon drawing of TAK including adenosine. The α -helices are labeled $\alpha 1$ through $\alpha 12$, and the β -strands are labeled $\beta 1$ – $\beta 14$. Figures 2–4 were drawn with Ribbons (Carson, 1987).

with O3'. There is a water molecule (W416) that hydrogen bonds to OD2 of Asp24; this could displace O3' if the ribose were tilted to approximately the same orientation as in HAK or RK.

The second adenosine binding site in HAK, which is thought to represent the ATP binding site, is not occupied in TAK. This site corresponds to the ATP-analog binding site in RK. Three of the residues that form contacts with this adenosine in HAK occur in the loop containing residues 286–295. This segment corresponds to residues 304–313 in TAK, which have some of the highest *B*-factors in the protein, suggesting considerable flexibility in the absence of substrate. In this regard, it is noteworthy that residues in the corresponding loop in RK also have very high *B*-factors.

Magnesium and chloride sites

While the human enzyme was crystallized at a high Mg^{2+} concentration (160 mM), the parasitic AK was crystallized in the presence of 2 mM Mg^{2+} , which is the optimum concentration for enzyme activity of the recombinant protein (Recacha et al., 2000). Three Mg^{2+} ions were present in the HAK structure, although only one of these appears to be important in enzymatic activity. The other two are involved in crystal packing contacts. One Mg^{2+} ion was located in the TAK structure, and it does not correspond to any of the three sites in HAK. It is ~ 25 Å from the adenosine and is coordinated to three carboxyl oxygen atoms (Ala185, Ile188, and Ala191) and two water molecules (W406 and W471) with distances ranging from 2.29 to 2.37 Å. It appears unlikely that this

Fig. 3. Superposition of TAK (green) and HAK (blue). The two adenosine molecules from the HAK structure are depicted as stick models.

Mg^{2+} ion represents the catalytic magnesium. Examination of $2F_o - F_c$ maps in the vicinity corresponding to the active site Mg^{2+} ion in HAK shows four water molecules that correspond to four of the six Mg^{2+} ligands in HAK, but there is no comparable electron density for the Mg^{2+} ion or the other two water molecules. One of the other two Mg^{2+} ions in HAK is partially coordinated to side-chain oxygen atoms from Asp130 and Asn131, which correspond to Lys134 and Glu135 in TAK. The other Mg^{2+} ion in HAK is coordinated to five water molecules and OG of Ser32; this residue corresponds to Phe38 in TAK.

The position of the chloride ion in TAK is very similar to the chloride ion in HAK that is close to adenosine. As in HAK, its closest contact with adenosine is with C2 of the adenine ring. There is no electron density that corresponds to the second chloride ion in HAK.

Discussion

The cDNA for TAK encodes a polypeptide of 363 amino acids, which is 18 residues longer than the human adenosine kinase. Several distinctive features in the primary sequence of TAK become easily identifiable when its sequence is compared to the enzyme from other sources (Fig. 1). There is a five-residue deletion in one of the four linking polypeptide segments, and there are two insertions of 11 and 5 residues. The larger of the two occurs between helices $\alpha 9$ and $\alpha 10$, and it is the most poorly defined region in the TAK structure. As a result of this insertion, the loop between these helices is much longer in the parasitic enzyme compared to other adenosine kinases, and helix $\alpha 10$ in *T. gondii* is three residues longer at the N-terminal end than its human counterpart. However, this region is on the opposite end of domain 1

Fig. 4. Space-filling models of HAK and TAK with bound adenosine. The adenosine molecule corresponding to adenosine-1 of the HAK structure is much more buried than adenosine in the TAK structure. Color-coding: carbon, green; nitrogen, blue; oxygen, red; sulfur, yellow; adenosine, magenta.

Fig. 5. Electron density ($2F_o - F_c$) map contoured at (1.0σ) showing the adenosine molecule and nearby residues.

Fig. 6. Stereoview of the adenosine-binding domain in TAK (magenta) superimposed on HAK (green). The TAK ligand is cyan, and the HAK ligand is black. Key TAK residues are numbered. The figure was drawn with CHAIN (Sack, 1988).

from the active-site cleft, and it is unlikely that it has any significant effect on enzymatic properties. The second insertion is between β 13 and β 14; it extends each β -strand by two residues and creates a slightly larger hairpin loop between them. As with the larger insertion, this structural change is distant from the active site and appears unlikely to have any effect on enzymatic activity. The most distinguishing feature of the TAK sequence is a deletion of five residues in the loop joining strand β 8 and helix α 5. This region occurs in one of the four polypeptide segments connecting the two domains in the protein. The three other polypeptides connecting these domains are of similar length in the human and parasitic forms. The shortening of this loop appears to result in a movement of domain 2 with respect to domain 1 in TAK. However, movement of the two domains because of the absence of ligand in the adenosine 5'-triphosphate (ATP) binding site cannot be excluded.

One of the two adenosine sites in HAK is postulated to represent the ATP binding site, based in part on its similarity to the ATP-analogue site in RK (Sigrell et al., 1998). Its only direct interaction with HAK is through hydrogen bonds with Gln289 at N1 and N6. The corresponding residue in TAK is Ala307, which cannot contribute similar hydrogen bonds. Eight residues interact indirectly with adenosine through hydrogen bonds with water molecules. Five of these utilize main-chain oxygen or nitrogen atoms, while the other three use side-chain atoms (Thr265 OG1, Thr271 OG1, and Asp286 OD2). These three residues correspond to Thr278, Val274, and Val304 in TAK, so two of the three side-chain interactions would not be available. In the structure of RK, there is no direct protein contact with the adenine moiety of the ATP analogue; all the hydrogen bonds are mediated through water molecules. Thus, the absence of these side chains in TAK would not preclude binding of ATP.

Most kinetic studies have suggested that the preferred order of substrate binding in adenosine kinase is adenosine prior to ATP (Palella et al., 1980; Rotllan & Miras Portugal, 1985; Hawkins & Bagnara, 1987; Bhaumik & Datta, 1988). The structure of HAK supports that hypothesis, since the adenosine binding site is significantly more buried and further from the solvent interface than the putative ATP-binding site. In TAK the presence of only one substrate molecule, occupying the same position as adenosine 1 in the HAK structure, is additional evidence for this order of substrate

binding. The lack of sequence homology in TAK and HAK for residues in the putative ATP binding site also suggests less specific interactions, and thus lower binding affinity between the protein and the second substrate molecule.

TAK is completely inactivated by $1 \mu\text{M}$ HgCl_2 (Recacha et al., 2000), suggesting that a thiol group is near the active site of the enzyme. The structure of HAK revealed a cysteine residue near the adenosine binding site (Cys123), but there was no cysteine near the putative ATP binding site. In TAK, there is also a cysteine residue near the adenosine binding site (Cys127). However, due to the movement of domain 2 relative to domain 1 in TAK, the sulfhydryl in TAK is about $3 \text{ \AA}$ further from the adenine ring and $0.5 \text{ \AA}$ further from the ribose, compared to HAK. There are no other cysteine residues close to the adenosine site or the putative ATP binding site.

As in other microbial adenosine kinases, as well as many other nucleoside and carbohydrate kinases, the sequence DTxGAGD (residues 312–318) is conserved, suggesting a critical role of this area in the activity of these kinases. In HAK, OD1 of Asp300 (corresponding to Asp318 in TAK) coordinates to Mg^{2+} through a water molecule, and OD2 coordinates to O5' of adenosine. In TAK there are similar hydrogen bonds to O5' and a water molecule (W495), even though no Mg^{2+} ion is present in the active site.

Materials and methods

Characterization of TAK

The expression and purification of TAK have been described in detail (Recacha et al., 2000). Enzymatically active recombinant protein expressed in *Escherichia coli* was monomeric as judged by size exclusion chromatography. The purified enzyme specifically utilized adenosine as its substrate but recognized nucleoside triphosphates other than ATP as a phosphate donor. A divalent cation such as Mg^{2+} or Mn^{2+} was essential for the activity of the enzyme, and low concentrations of EDTA blocked the activity. The enzyme was also very sensitive to Hg^{2+} ion, indicating the presence of a thiol group at or near the active site.

Crystallization and preliminary X-ray diffraction analysis of TAK

Crystals of TAK suitable for X-ray diffraction were grown at room temperature using ammonium sulfate as precipitant at pH 8.5 (Recacha et al., 2000). Although the protein crystallized with or without the addition of adenosine, the best crystals were grown when the crystallization medium contained $20 \mu\text{M}$ adenosine and 2 mM MgCl_2 . Crystals grew to the maximum dimension of $0.2 \times 0.4 \times 0.2 \text{ mm}$ in three to four days. The size and shape of the crystals were improved considerably on addition of up to 3% (v/v) glycerol in the crystallization medium. Diffraction data from a crystal soaked in a cryoprotectant solution containing 25% glycerol (v/v) were collected at -170°C using an X-stream low temperature device on an R-axis IV image plate. Intensity data were processed using the HKL package (Otwinowski & Minor, 1997). The crystals belong to space group $P2_1$ with $a = 47.6 \text{ \AA}$, $b = 68.8 \text{ \AA}$, $c = 57.3 \text{ \AA}$, and $\beta = 100.3^\circ$. There is one molecule in the asymmetric unit, and the value of V_m (Matthews, 1968) is 2.41, which corresponds to a solvent volume fraction of 49%.

A total of 33,098 independent reflections (98.6% complete) were collected at $1.8 \text{ \AA}$ resolution with average $\langle I/\sigma \rangle$ of 14.0 and R_{sym} of 4%. Table 2 gives statistics for the data processing.

Table 2. Data processing statistics for *T. gondii* adenosine kinase

Resolution limit (Å)	1.80
Total number of observations	317,522
Number of unique reflections	33,084
Completeness (%)	98.6
Redundancy	9.6
Overall I/σ	13.2
R_{merge} (%)	4.3
Resolution of the highest resolution shell (Å)	1.80–1.86
Completeness in the highest resolution shell (%)	89.3
Reflections with $I/\sigma > 3$ in the highest resolution shell (%)	55.0
R_{merge} for the highest resolution shell (%)	21.0

Structure determination and refinement

The crystal structure of adenosine kinase was solved using the molecular replacement routines in CNS (Brünger et al., 1998). For the cross-rotation search, the 1.5 Å crystal structure of human adenosine kinase (Mathews et al., 1998; Protein Data Bank (PDB) entry 1BX4) was used as the search model. HAK and TAK share about 42% similarity in their amino acid sequences, so the original search model was constructed by substituting alanine for nonidentical residues. Based on the proposed sequence alignment, residues 10–44, 54–159, 161–185, 187–236, 239–301, and 307–363 (TAK numbering) were included. This model gave a strong single peak in the cross-rotation and translation functions, but the packing analysis showed interpenetration involving residues from the domain 2. Residues in these regions were deleted, but repeated attempts to refine this partial structure were unsuccessful. Furthermore, the difference Fourier maps did not show clear density for any of the missing regions. A comparison of the structure of HAK with the homologous structure ribokinase (Sigrell et al., 1998) revealed a very conserved core structure, but there were a number of large differences in some of the loops. Sequence alignment with TAK also suggested that there might be significant movement of domain 1 with respect to domain 2 compared to the structure of HAK, since there is a five-residue deletion in one of the linker regions between the two domains. Therefore, a new search model consisting only of core residues from domain 2 was constructed. This model included residues 14–27, 63–82, 87–145, 161–182, 192–227, 245–253, 273–297, and 315–315 (TAK numbering). A cross-rotation function for this model was calculated using data between 15 and 4 Å resolution. The rotation search values showed a clear maximum; the highest RF-function value was 0.1589, while the next highest value was only 0.0608. All of the peaks from the rotation search (194 peaks) were subjected to Patterson correlation refinement (Brünger, 1990), treating the entire molecule as a rigid body, and then translation functions were calculated using data from 15 to 4 Å. The highest translation function T value was 0.307, and this solution was correct.

Examination of a $2F_o - F_c$ map using this solution showed several regions of poor density around the model, so those regions were deleted, and the model was subjected to simulated annealing using torsion angle dynamics, using all data to 1.8 Å resolution, the maximum likelihood target, and a bulk solvent correction. All model refinement was carried out using the program CNS (Brünger et al., 1998) with the stereochemical parameters defined by Engh

and Huber (1991). No sigma cutoff was applied to the data. Five percent of the data were randomly selected and removed prior to refinement for analysis of the free R -factor (Brünger, 1992). The R -factor for this model (224 residues) was 0.422, and R_{free} was 0.448.

To reduce model bias in subsequent electron density maps, density modification by solvent flipping and density truncation was used (Abrahams & Leslie, 1996). This yielded much better maps than simple $2F_o - F_c$ maps at the early stages of refinement and rebuilding. Electron density maps were displayed and model coordinates were fitted using the program CHAIN (Sack, 1988). The progress of the refinement was guided by the decrease in both the conventional and free R -factors.

An overall B -factor for the initial model was calculated and used as the B -factor for each atom in the initial stages of refinement. The entire chain was fit, except for the loop containing residues 238–245, because there was essentially no electron density in this region. The electron density clearly showed that Pro12 adopted a *cis* conformation. Early in the course of the refinement, we noted that there was significant electron density between Cys340 and Cys363, which could be modeled by a disulfide bond between these two residues. The sequence of HAK has no corresponding cysteine residues, and there are no disulfide bonds in that structure.

Multiple cycles of manual rebuilding and simulated annealing eventually reduced the R -factor to 0.292 (R_{free} 0.319). At this stage individual B -factor refinement was begun, and water molecules were introduced using the water picking routine from CNS that searched the peaks in the $2F_o - F_c$ map and checked distance criteria for reasonable hydrogen-bond donors and acceptors. Based on analysis of the B -factors and the coordination geometry, one water molecule was changed to a magnesium ion, and another was changed to a chloride ion. There was also a strong triangular-shaped density that was modeled as an acetate ion. Hydrogen bonds and short contacts involving adenosine are listed in Table 3.

Careful analysis of the $2F_o - F_c$ maps showed two discrepancies from the expected protein sequence. Electron density maps showed additional density extending beyond the side chain of Leu179. DNA sequence analysis, using both strands of the DNA of the construct, showed phenylalanine at this position instead of leucine, as determined from the cDNA sequence. The B -factors of atoms in this side chain after refinement were comparable to those for other residues in this region of the structure. This mutation may have occurred during amplification of the coding sequence from the cDNA using Taq DNA polymerase. Residual density in the vicinity of Cys202 could not be modeled by additional water molecules because of the short distance from the cysteine S atom. The identity of this residue as cysteine was confirmed by DNA sequence analysis, and we are not sure what this density represents. It is probably a trace contaminant from one of the reagents used for purification and crystallization, which binds to the reactive thiol group.

Superposition of the HAK structure on the TAK structure showed very similar geometry of the adenosine-binding residues, and there was ill-defined density at the suspected adenosine site, especially in the probable ribose position. An adenosine molecule was fit to the experimental density, but since it seemed likely that the adenosine site was only partially occupied in the structure, the occupancies of these atoms were refined as a group following positional refinement. Simply refining the occupancies for these 19 atoms lowered the R_{free} by 0.4%. After additional positional refinement, the adenine moiety refined to a reasonable position with protein

Table 3. Hydrogen bonds and close contacts involving adenosine (<3.45 Å)

Adenosine atom	Protein atom	distance
N1 Asn20 CB		3.29
N1 Asn20 ND2		2.96
C2 Asn20 CB		3.30
C2 Asn20 O		3.15
C2 Cl ¹⁻		3.14
N3 Asn20 O		3.45
N3 Gly69 CA		2.90
N3 Gly69 C		3.49
N3 Ser70 N		3.11
N6 Tyr169 CG		3.45
N6 Tyr169 CD1		3.39
C8 W517		2.99
C8 W536		3.21
N9 W517		3.26
N9 W536		3.28
C1' Gly69 CA		3.31
C1' Gly69 N		3.48
C1' W517		3.38
C2' W416		3.48
C2' W517		2.59
O2' Asp24 OD1		2.75
O2' W416		2.75
O2' W517		3.27
C4' W416		2.98
O4' Gly69 CA		3.41
O4' Ser70 N		3.34
O4' W416		3.36
O4' W536		3.08
C5' W536		2.95
O5' Asp318 OD2		2.53
O5' W536		3.07

contacts very similar to the adenine portion of adenosine in the HAK structure. However, the ribose ring refined to a slightly different position, and only four hydrogen bonds that corresponded to the hydrogen-bonding pattern in HAK were noted.

The final model includes 346 residues (10–237 and 246–363), adenosine, one Mg²⁺ ion, one chloride ion, one acetate ion, and 142 water molecules. The *R*-factor is 0.214 (*R*_{free} = 0.233) with tightly restrained geometry (Table 1). The atomic coordinates and structure factors for TAK have been deposited with the Protein Data Bank (RCSB number RCSB010069; PDB number 1DGM).

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